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Electroporation-mediated gene transfer of *SOX trio* to enhance chondrogenesis in adipose stem cells

G.-I. Im*, H.-J. Kim

Department of Orthopaedics, Dongguk University Ilsan Hospital, Republic of Korea

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SUMMARY

Objective: The aim of the present study was to determine if the electroporation-mediated gene transfer of *SOX trio* enhances the chondrogenic potential of adipose stem cells (ASCs).
Design: ASCs were transfected with *SOX trio* genes using an electroporation technique and cultured for 3 weeks. The pellets were analyzed for DNA and glycosaminoglycan (GAG) analysis, and the gene and protein expression of SOX-5, SOX-6, SOX-9, type 1 collagen (COL1Al), type 2 collagen (COL2Al) and type 10 collagen (COL10A1) using real-time PCR and Western blot analysis. Further *in vivo* studies were carried out by subcutaneous transplantation of pellets in severe combined immunodeficiency (SCID) mice for 3 weeks.
Results: The gene transfer efficiency was high (approximately 70%). Transfected ASCs showed high expression of corresponding genes after 21 days, and each SOX protein was detected in ASCs transfected with the corresponding gene. The chondrogenic differentiation of ASCs, as demonstrated by GAG levels and Safranin-O staining, showed significant enhancement when *SOX trio* were co-transfected, while subsets with single gene transfer of *SOX-5*, *-6*, or *-9* did not show significant elevation. *SOX trio* co-transfection enhanced *COL2A1* mRNA, but did not increase *COL1A1* and *COL10A1* mRNA. Type II collagen protein dramatically increased, and type X collagen decreased with co-transfection of the *SOX trio*. When pellets were implanted in the subcutaneous pouch of SCID mice for 3 weeks, ASCs co-transfected with *SOX trio* demonstrated abundant proteoglycan, significantly reduced mineralization.
Conclusion: The electroporation-mediated transfection of *SOX trio* greatly enhances chondrogenesis from ASCs, while decreasing hypertrophy.

Introduction

Injury of articular cartilage (AC) in adults usually leads to osteoarthritic changes of the joint because AC has a limited capacity for self-repair[1]. Cell-based therapy is currently the mainstay of efforts to regenerate AC. However, the use of autologous chondrocytes that have been popularized in recent decades has some limitations, including donor site morbidity and dedifferentiation of chondrocytes[2,3]. These shortcomings have prompted further investigations to find other sources that would offer an abundant number of self-replenishing cells[4,5].

Mesenchymal stem cells (MSCs) have a pluripotential capacity, which allows them to differentiate into a variety of connective tissue lineages, including bone, cartilage, fat, and muscle[6–9]. The potential of MSCs for self-replication and differentiation provides therapeutic opportunities for the treatment of musculoskeletal defects. Although bone marrow is the best known source of MSCs, adipose tissue offers the most abundant and easily accessible pool of MSCs[10–12]. Adipose stem cells (ASCs) obtained from lipoaspirates have multi-lineage potential to differentiate into adipogenic, chondrogenic, myogenic, and osteogenic cells[11,13,14]. However, several studies, including one from our group, noted that ASCs had lower chondrogenic potential using a combination of growth factors currently known to induce chondrogenesis of MSCs from bone marrow[15–18]. Despite the reportedly poor response of ASCs to chondrogenic signals, ASCs merit further investigation because of their acquisition advantage. We had investigated the conditions for growth factor application, finding that either high-dose transforming growth factor (TGF)-β plus insulin-like growth factor (IGF)-I or a combination of TGF-β plus bone morphogenetic protein (BMP)-7 was effective in inducing chondrogenic differentiation of ASCs[19,20]. One of the dilemmas in inducing chondrogenesis from

* Address correspondence and reprint requests to: Gun-Il Im, Department of Orthopaedics, Dongguk University Ilsan Hospital, 814 Siksa-Dong, Goyang 411-773, Republic of Korea. Tel: 82-31-961-7315; Fax: 82-31-961-7314.
E-mail address: gunil@duih.org (G.-I. Im).

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ASCs is the simultaneous increase of types I and X collagen, which signifies inadequate differentiation and hypertrophy, respectively.

Several lines of evidence indicate the significance of SOX transcription factors for the maintenance of chondrocytic phenotypes[21,22]. These factors belong to a family of regulatory molecules related to sex-determining factor SRY (sex-determining region Y)[23], which is expressed in all chondrogenic cells, except hypertrophic chondrocytes[21]. SOX-9 binds and activates other chondrocyte-specific enhancer-elements in *COL2A1*, *COL9A1*, *COL11A2*, and *aggrecan in vitro*[24–27]. Two other members of the SOX family (SOX-5 and SOX-6) are also required for perfect chondrogenesis, which are co-expressed with SOX-9 in all chondroprogenitors and differentiated chondrocytes, and cooperated with SOX-9 to activate *COL2A1* gene[21,28,29]. Previous studies have also reported on *SOX* gene transfers to induce chondrogenesis in human embryonic stem cells and fibroblast, and to promote chondrogenesis from murine MSCs[30,31]. However, the effect of *SOX trio* gene transfer on human ASCs has not been reported.

With these limitations of growth factors in inducing chondrogenesis from ASCs, and previous investigations reporting enhanced chondrogenesis and inhibition of hypertrophy in a variety of cells by gene transfer of *SOX trio*, we reasoned that gene transfer of these genes would enhance chondrogenesis from ASCs. As viral gene transfer methods are associated with the risk of immunologic reactions and mutagenesis[32–34], these methods are not indicated for the treatment of non-lethal disease. As the purpose of gene transfer of the *SOX trio* is for cartilage tissue engineering, non-viral methods should be used if considered for clinical applications. Thus, in this study, we tested the hypothesis that non-viral transfection of the *SOX trio* gene would significantly enhance the chondrogenic potential of ASCs. We used a microporation method, which has a capillary tip and pipette-based gene transfer technique[34]. This technique is known to circumvent a number of harmful effects of a cuvette-based electroporation method, such as pH variation, temperature rising, turbulence, and metal ion generation[35]. Microporation provides an easier and more efficient method for hard-to-transfect cells with its high transfection efficiency and reproducibility[35]. Therefore, the present study was embarked upon an endeavor for electroporation-mediated non-viral gene transfer of *SOX trio* in human ASCs.

Materials and methods

Sample procurement

The adipose tissue samples were isolated from the lipoaspirates generated during elective liposuction procedures on five patients (mean age, 41 years; range, 36–55 years). This study was approved by the Institutional Review Board at our university, and informed consent was obtained from all the individuals included in the study.

Cell isolation and cultivation

ASCs were isolated from lipoaspirates, then expanded as described in previous studies[11,17]. Cells were cultured in Dulbecco's modified Eagle medium (DMEM)/F-12 supplemented with 10% fetal bovine serum (FBS; Invitrogen/GIBCO, Grand Island, NY, USA) and 1% antibiotics, and maintained at 37°C for 48 h in a 5% CO_2 humidified atmosphere. Cells were then washed with phosphate buffered saline (PBS; Welgene, Daejeon, Korea) to remove non-adherent material. During the expansion period, medium was replaced twice per week. When cells reached 80% confluence, they were detached from culture dishes using 0.25% trypsin containing 1 mM ethylenediaminetetraacetic acid (EDTA; Gibco BRL Green Island, NY, USA) washed with PBS, counted, and re-plated. After culturing through passage 2, cells were suspended in a cryopreservation medium containing 90% FBS and 10% dimethylsulfoxide.

Construction of plasmids

The coding regions of human *SOX-5*, *SOX-6*, and *SOX-9* from the NIH Mammalian Gene Collection (MGC) cDNA Clones (Invitrogen Corporation, Carlsbad, CA, USA) were amplified by polymerase chain reaction (PCR) and cloned into *pECFP-C1* expression vectors (Clontech, Palo Alto, CA, USA). In order to increase cloning efficiency, the 5′ end was made sticky and the 3′ end was made blunt. After PCR, the insert was prepared using enzymes (*Sma*I and *Bgl*II; Takara Bio Inc., Otsu, Japan). *Sma*I was applied to digest the 3′ end, followed by T7 polymerase (Takara Bio Inc.), which induced the blunt end. *Bgl*II was used to produce a sticky 5′ end. For the vector, *Sma*I and *Bgl*II were used for the same purpose. The prepared vector and insert underwent ligation using Ligation Mix (Takara Bio Inc.), and underwent transformation to obtain the respective clones. The PCR products were verified by DNA sequencing.

Transfection by electroporation

Sub-confluent ASCs were harvested and washed with PBS. Cells were resuspended in resuspension buffer R at a density of 3×10^5 cells/ml and mixed with 0.5 μg plasmids. Then, electroporation was performed with a Microporator (Invitrogen) using programs recommended by the manufacturer (1400 V for 20 ms). After electroporation, cells were plated onto a 12-well plate and placed at 37°C in 5% CO_2. Six subsets were prepared, as follows: #1, ASCs transfected with *pEGFP-C1* without interposed genes of transcription factors (negative control); #2, ASCs transfected with *pEGFP-C1* interposed with *SOX-5* gene; #3, ASCs transfected with *pEGFP-C1* interposed with *SOX-6* gene; #4, ASCs transfected with *pEGFP-C1* interposed with *SOX-9* gene; #5, ASCs transfected with one-third doses (0.17 μg) of each plasmid (*SOX-5*, *-6*, *-9*); #6, ASCs treated with a combination of 5 ng/ml of TGF-ß$_2$ and 100 ng/ml of BMP-7 (positive control group), which are based on our previous study[19].

Confirmation of gene transfer efficiency using fluorescence microscopy and flow cytometry

After performing gene transfer, the transfection efficiency of each gene was confirmed using fluorescence microscopy (Leica DMI 6000B; Wetzlar, Germany) and flow cytometry (Beckman Coulter Inc., Fullerton, CA, USA) 24 h after transfection. For flow cytometry, the cells were thrice washed and suspended in a media consisting of Hank's Buffered Salt Solution (HBSS; Welgene) with 2% FBS (Invitrogen/GIBCO). The ASCs that were not used for gene transfer were used as the negative control. Thereafter, the cells were analyzed using CXP software (Beckman Coulter Inc.).

Pellet culture

Transfected ASCs were cultured in DMEM/F-12 supplemented with 1% insulin-transferrin-selenium (ITS), 10^{-7} M dexamethasone, 50 μM ascorbate-2-phosphate, 50 μM L-proline, and 1 mM sodium pyruvate. For pellet cultures, 2.5×10^5 cells suspended in 500 μl of culture medium were aliquoted into 15 ml polypropylene centrifuge tubes, and spun in a benchtop centrifuge at 500*g* for 10 min. The tubes were then placed in an incubator at 37°C in a humidified 95% air/5% CO_2 atmosphere. The medium was changed every third day. The pellet culture was carried up to 3 weeks.

DNA quantification and glycosaminoglycan (GAG) analysis

The pellets of each group were digested overnight in papain buffer at 60°C. The DNA content was determined using the Quant-iT™ dsDNA assay kit and the Qubit Fluorometer system (Invitrogen). GAG production was determined using a Blyscan Kit (Biocolor, Carrickfergus, UK), according to the manufacturer's instructions. GAG levels were expressed as micrograms of GAG per microgram of DNA.

Reverse transcription and real-time PCR analysis

Total RNA from cells were isolated with an RNeasy Mini Kit (Quiagen, Hilden, Germany), according to the manufacturer's instructions, then quantified using the Quant-iT™ RNA assay kit and the Qubit Fluorometer system (Invitrogen). All total RNA samples were treated with DNAse I (Quiagen). Total RNA was reverse-transcribed with Multiscribe reverse transcriptase (Invitrogen) oligo (dT) primer in a 40 μl reaction volume, according to the manufacturer's instruction. All the PCR reactions were performed on the LightCycler 480 system® (Roche Diagnostics, Mannheim, Germany) in standard 10 μl reactions, as follows: 4.5 μl (10 ng) cDNA, 0.5 μl of 10 μM sense primer, 0.5 μl of 10 μM antisense primer and 4.5 μl LightCycler 480 SYBR Green I Master mix (Roche Diagnostics). The primers and conditions for *SOX-5*, *SOX-6*, *SOX-9*, type 1 collagen (*COL1A1*), type 2 collagen (*COL2A1*), and type 10 collagen (*COL10A1*), are listed in Table I. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control for PCR amplification and the relative normalization ratio of PCR products derived from each target gene was calculated using software of the LightCycler System. All experiments were performed in triplicate.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

To confirm the translation of SOX-5, -6, and -9 and chondrogenic gene expression at the protein level, protein extracts were analyzed by Western blotting for SOX-5, SOX-6, SOX-9, and types I, II and X collagen. Briefly, cells were washed twice with cold PBS, and suspended in 50 μl of radioimmunoprecipitation assay (RIPA) lysis solution (Pierce, Rockford, IL, USA), according to the manufacturer's instructions. The protein concentration was determined using a Qubit assay kit (Invitrogen) and equal amount of protein extracts was fractionated by 10% SDS-PAGE (Bio-Rad, Hercules, CA, USA), and transferred onto a BioTrace™ NT Nitrocellulose Transfer Membrane. After blocking with Tris-buffered saline (TBS)-T (10 mM Tris; 150 mM NaCl, and 0.05% Tween-20) containing 3% non-fat powdered milk (Bio-Rad Laboratories, Inc, Gothenburg, Sweden), the blots were incubated with specific primary antibody for 2 h at room temperature, washed, and incubated with secondary antibody for 1 h at room temperature, then washed again. The blots were developed using the SuperSignal® West Pico Chemiluminescent Substrate (Pierce) following the manufacturer's protocols. After three washes, the protein bands were visualized with an enhanced chemiluminescence (ECL) Western blot analysis system (Amersham Biosciences, Piscataway, NJ, USA). Primary antibodies were rabbit type I collagen polyclonal antibody (Millipore, Billerica, MA, USA), mouse type II collagen monoclonal antibody (Millipore), mouse anti-human type X collagen monoclonal antibody (Sigma–Aldich, St. Louis, MO, USA) and rabbit SOX-5, SOX-6, and SOX-9 polyclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) diluted 1:200 in TBS-T/2.5% non-fat dry milk. Secondary antibody was horseradish peroxidase (HRP)-conjugated goat anti-rabbit, goat anti-mouse IgG antibody diluted 1:2000 (Santa Cruz Biotechnology, Inc.) in TBS-T/2.5% powdered non-fat dry milk. This experiment was repeated in three samples, each from different persons.

Table I
Primers used for real-time PCR

Gene symbol	Sequences (5′–3′)	Accession no.
COL1A1	F-CCGCCGCTTCACCTACAGC	NM_000088
	R-TTTTGTATTCAATCACTGTCTTGCC	
COL2A1	F-ATAAGGATGTGTGGAAGCCG	NM_001844
	R-TTTCTGTCCCTTTGGTCCTG	
COL10A1	F-CAGTCATGCCTGAGGGTTTT	NM_000493
	R-GGGTCATAATGCTGTTGCCT	
SOX-5	F-CAAGGCAATCCAAGAAGCTC	AB_081589
	R-CCAATCATTGCATGGCTAAA	
SOX-6	F-AGGATCTCGCTGGAAATCAA	AF_309034
	R-CTGCCTCATCTCCTGTCTCC	
SOX-9	F-GGAGCTCGAAACTGACTGGAA	NM_000346
	R-GAGGCGAATTGGAGAGGAGGA	
GAPDH	F-CACATGGCCTCCAAGGAGTAA	NM_002046
	R-GTACATGACAAGGTGCGGCTC	

Subcutaneous transplantation in severe combined immunodeficiency (SCID) mice

Pellets of subsets #1 and #5 ($n = 3$) were cultured as an *in vitro* pellet culture for 7 days, then transplanted into subcutaneous pockets in the upper dorsal area of anesthetized SCID mice. Pellets were attached to a non-absorbable surgical suture fixed with fibrin glue to facilitate recovery at the time of harvest. Six subcutaneous pouches prepared on the backs of each male SCID mice. Samples were explanted 3 weeks after implantation, and analyzed for GAG, calcium contents, and histology.

Measurement of calcium content from harvested pellets

The harvested pellets of each group were added with 100 μl of lysis buffer (RIPA; Thermo, Rockford, IL, USA) and homogenized with liquid nitrogen. The lysate was maintained for 2 h, then centrifuged for 20 min at 12,000 rpm in a microcentrifuge. Calcium Colorimetric Assay Kit (Biovision, Mountain View, CA, USA) was used to measure the calcium contents from the supernatant according to the manufacturer's instructions. This assay utilizes the formation of chromogenic complex formed between calcium ions and O-cresolphtalein. The measured contents were normalized to the DNA contents of the pellets.

Histology

Pellets cultured *in vitro* for 3 weeks or harvested from the *in vivo* study were fixed in 4% paraformaldehyde solution for 3 h, then dehydrated with 100% ethanol, washed with xylene, and embedded with paraffin. Sections with a thickness of 4 μm were cut from the paraffin block and coated on the glass slide. Safranin-O staining for proteoglycan, and Alizarin red staining for mineralization were performed. For Safranin-O staining, sections were deparaffinized with xylene and ethanol, treated with aqueous Safranin-O (0.1%) for 30 min, and washed with distilled water. For Alizarin red staining, specimens were stained with 2% Alizarin red solution (Junsei Chemical, Tokyo) for 10 min, and washed with distilled water.

GenBank sequences

Human gene sequences were obtained from GenBank (accession nos. AB081589 for *SOX-5*, AF309034 for *SOX-6*, and Z46629 for *SOX-9*).

Statistical analysis

All quantitative data are expressed as the group means and 95% confidence intervals. Statistical analysis was performed using analysis of variance followed by Dunnett's correction for multiple comparisons and Mann–Whiney *U* test using SPSS software (SPSS, Inc., Chicago, IL, USA). Significance was set at a $P < 0.05$.

Results

Transfection of SOX-5, -6, and -9 into ASCs

The efficiency of transfection was investigated by fluorescence microscopy and fluorescence-activated cell sorting (FACS) analysis 24 h after microporation. The gene transfection efficiency was high for all groups; greater than two-thirds of cells were transfected [Fig. 1(A), Table II]. The fluorescence was detectable in the cultured pellet 21 days after transfection [Fig. 1(B)].

Expression of the SOX trio gene and protein

In order to determine whether or not expressions of the transfected genes and proteins still occur after a 3-week *in vitro* pellet culture, real-time PCR and Western blotting were performed. Even after 21 days, ASCs transfected with *SOX-5*, *SOX-6*, or *SOX-9* had a 100- to 550-fold greater gene expression of *SOX-5*, *SOX-6*, or *SOX-9* when compared with the control ($P < 0.0001$, <0.0001, $=0.011$ respectively). When the *SOX trio* were co-transfected, each gene was expressed in a greater amount than in the control ($P = 0.041$, 0.023, 0.045 respectively), although the level of expression was lower than in single gene transfer [Fig. 2(A)]. The SOX-5, SOX-6, and SOX-9 protein was clearly detected in ASCs with single transfection of each gene, and more weakly detectable in ASCs with co-transfection of the *SOX trio*. These results showed that the transfected genes were continuously expressed up to 3 weeks of *in vitro* culture [Fig. 2(B)].

Table II
The transfection efficiency of each subset

Transfected gene	Vector	*SOX-5*	*SOX-6*	*SOX-9*	*SOX-5, -6, -9*
Mean percentage of transfected cells [95% CI]	83.7 [77.9, 89.5]	76.7 [68.9, 84.5]	66.7 [56.3, 75.1]	74.9 [71.1, 78.7]	76.8 [73.8, 79.8]

DNA and GAG levels

The amount of DNA and GAG was measured to assess the cell number and the production of chondroid extracellular matrix. The DNA content of all groups was between 0.4 and 0.6 μg, with no significant difference in the DNA levels among subsets. These results indirectly showed that gene transfection did not have a significant effect on cell number [Fig. 3(A)]. GAG level normalized to DNA significantly increased by 4.2-fold when *SOX trio* genes were co-transfected ($P = 0.007$) and by 3.8-fold when pellets were treated with TGF-β_2 and BMP-7 ($P = 0.015$). Other subsets with single gene transfer of *SOX-5*, *-6*, and *-9* did not show a significant elevation in the GAG level normalized to DNA [Fig. 3(B)]. Safranin-O staining, which detects proteoglycan synthesis, showed intense metachromasia with *SOX trio* co-transfection, corroborating the results of the GAG analysis [Fig. 3(C)]. The results above show that the chondrogenic differentiation of ASCs as demonstrated by GAG synthesis is enhanced only when the *SOX trio* genes are co-transfected.

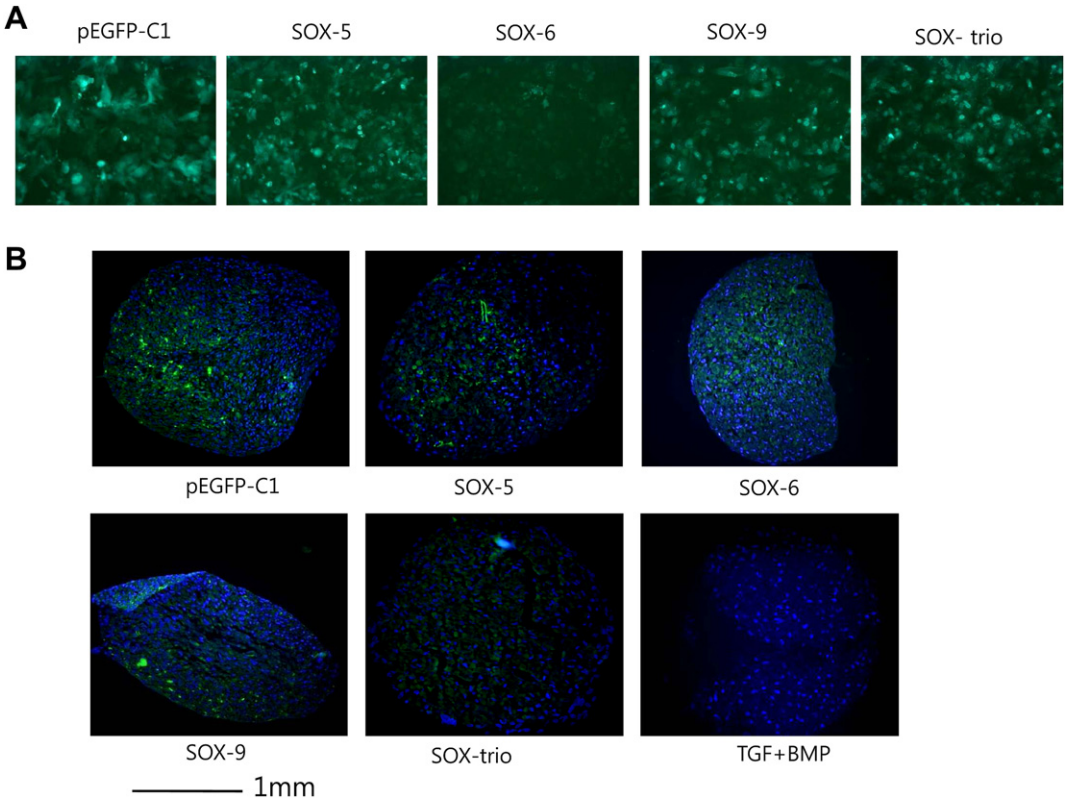


Fig. 1. Transfection was demonstrated by fluorescence microscopy. ASCs transfected with *pEGFP-C1* without interposed gene, *SOX-5*, *SOX-6*, *SOX-9*, and all three genes (*SOX trio*): 24 h after transfection (A) and 21 days after transfection and pellet culture (B). 4',6-diamidino-2-phenylindole (DAPI) staining (blue, indicating nuclei) was merged with green fluorescent protein (GFP) fluorescent imaging (green).

A mRNA normalized ratio

Gene	SOX-5		SOX-6		SOX-9	
N=3	Mean fold change [95% CI]	P-value vs control	Mean fold change [95% CI]	P-value vs control	Mean fold change [95% CI]	P-value vs control
Vector	1		1		1	
SOX-5	173.14 [124.51, 221.77]	**<0.0001**	2.20 [0.93, 3.47]	1.000	1.51 [0.86, 2.15]	1.000
SOX-6	1.08 [0.01, 2.16]	1.000	97.12 [76.84, 117.40]	**<0.0001**	1.62 [0.09, 3.14]	1.000
SOX-9	1.92 [0.99, 3,90]	1.000	4.2 [-2.79, 11.19]	0.982	544.39 [-159.67, 1248.45]	**0.011**
SOX-trio	45.73 [29.6, 61.9]	**0.041**	22.8 [16.15, 29.37]	**0.023**	76.00 [-13.58, 165.60]	**0.045**
TGF-β+BMP-7	0.97 [0.10, 1.85]	1.000	2.14 [0.16, 4.12]	1.000	1.69 [0.10, 3.29]	1.000

B

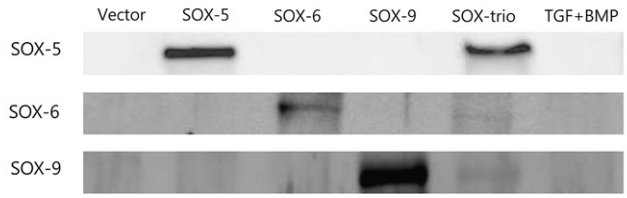


Fig. 2. Transfection of *SOX-5*, *-6*, and *-9* elevated mRNA and protein expression of transfected gene. Gene (A) and protein (B) expression of *SOX-5*, *SOX-6*, and *SOX-9* in ASCs transfected with *pEGFP-C1* without interposed gene (vector), *SOX-5*, *SOX-6*, *SOX-9*, and all three genes (*SOX-5*, *-6*, and *-9*), and in ASCs treated with TGF-β2 and BMP-7 (TGF + BMP) after 21 days of *in vitro* culture. $N = 3$, CI: confidence interval.

Expression of chondrogenic marker genes

Gene expression of *COL2A1*, the chondrogenic marker, significantly increased by nine-fold when all three *SOX* genes were co-transfected ($P = 0.045$) and by seven-fold when pellets were treated with TGF-β2 and BMP-7 ($P = 0.145$). Transfection of a single gene transfer of *SOX-5*, *-6*, or *-9*, however, was not as effective in inducing chondrogenic differentiation of ASCs [Fig. 4(A)]. *COL1A1* mRNA expression, which signifies inadequate differentiation, significantly increased with the treatment of TGF-β2 and BMP-7 ($P = 0.015$), while not changing significantly in other subsets, including *SOX trio* co-transfection [Fig. 4(A)]. *COL10A1* gene

A DNA

N=5	Mean DNA contents (μg) [95% CI]	P-value vs control
Vector (control)	0.44 [0.27, 0.61]	
SOX-5	0.43 [0.27, 0.60]	1.000
SOX-6	0.57 [0.42, 0.73]	0.285
SOX-9	0.49 [0.39, 0.58]	0.662
SOX-trio	0.51 [0.37, 0.65]	0.754
TGF-β+BMP-7	0.53 [0.37, 0.70]	0.741

B GAG/DNA

N=5	Mean GAG / DNA (μg/μg) [95% CI]	P-value vs control
Vector (Control)	2.79 [0.01, 5.58]	
SOX-5	3.34 [0.23, 6.45]	0.998
SOX-6	1.85 [1.14, 2.57]	1.000
SOX-9	3.28 [2.53, 4.02]	0.452
SOX-trio	6.92 [4.83, 9.00]	**0.007**
TGF-β+BMP-7	6.25 [4.41, 8.08]	**0.015**

C

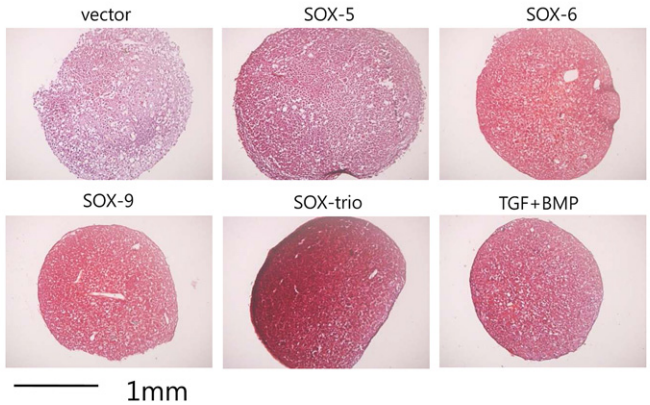


Fig. 3. DNA level was unaffected while GAG was elevated by *SOX trio* co-transfection in ASCs. DNA levels (A), GAG levels normalized to DNA (B), and Safranin-O staining of ASCs (C) transfected with *pEGFP-C1* without interposed gene (vector), *SOX-5*, *SOX-6*, *SOX-9*, and all three genes (*SOX trio*), and in ASCs treated with TGF-β2 and BMP-7 (TGF + BMP) after 21 days of *in vitro* culture. $N = 5$, CI: confidence interval.

A mRNA normalized ratio

Gene	Type II collagen		Type I collagen		Type X collagen	
N=3	Mean fold change [95% CI]	P-value vs control	Mean fold change [95% CI]	P-value vs control	Mean fold change [95% CI]	P-value vs control
Vector (Control)	1		1		1	
SOX-5	1.68 [0.56, 2.81]	0.999	0.86 [0.71, 1.01]	1.000	2.54 [0.86, 4.21]	0.801
SOX-6	0.67 [-0.15, 1.49]	1.000	2.42 [0.08, 4.77]	0.355	2.23 [-0.79, 5.26]	0.901
SOX-9	1.91 [-0.28, 4.10]	0.996	2.12 [1.00, 3.23]	0.565	1.71 [0.34, 3.08]	0.988
SOX-trio	8.89 [2.28, 15.49]	**0.045**	1.00 [0.36, 1.64]	1.000	1.35 [0.79, 1.91]	1.000
TGF-β+BMP-7	7.07 [1.16, 12.97]	0.145	4.03 [2.88, 5.18]	**0.015**	6.34 [2.23, 10.45]	**0.024**

B

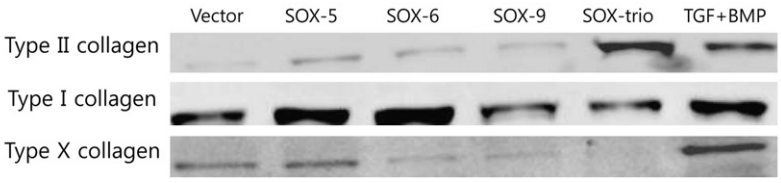


Fig. 4. Chondrogenic marker genes and proteins were enhanced by *SOX trio* co-transfection. Gene expression of *COL2A1*, *COL1A1*, and *COL10A1* (A), Western blotting for type II collagen, type I collagen and type X collagen (B) in ASCs transfected with *pEGFP-C1* without interposed gene (vector), *SOX-5*, *SOX-6*, *SOX-9*, and all three genes (*SOX trio*), and in ASCs treated with TGF-β2 and BMP-7 (TGF + BMP) after 21 days of *in vitro* culture. $N = 3$, CI: confidence interval.

expression was significantly increased by six-fold when treated with TGF-β2 and BMP-7 ($P = 0.024$), but did not change with *SOX trio* co-transfection [Fig. 4(A)]. These gene expression results show that *SOX trio* co-transfection enhances *COL2A1*, while not increasing *COL1A1* and *COL10A1*, as occurs with TGF-β2 and BMP-7 treatment.

Enhancement of chondrogenic marker protein by SOX trio co-transfection

Protein expression of types II, I, and X collagen was confirmed using Western blotting. Type II collagen dramatically increased with co-transfection of *SOX trio*, which was greater than induced with TGF-β2 and BMP-7 treatment. Type I collagen was detected in all subsets, and increased in ASCs transfected with *SOX-5* and *SOX-6*, and in ASCs treated with TGF-β2 and BMP-7. Type X collagen expression was decreased in ASCs with *SOX trio* co-transfection while it remarkably increased with TGF-β2 and BMP-7 treatment [Fig. 4(B)]. These results of protein expression further confirmed that *SOX trio* co-transfection increases chondrogenesis and blocks hypertrophy of ASCs.

Persistent proteoglycan and reduced calcification in in-vivo transplanted ASCs co-transfected with SOX trio

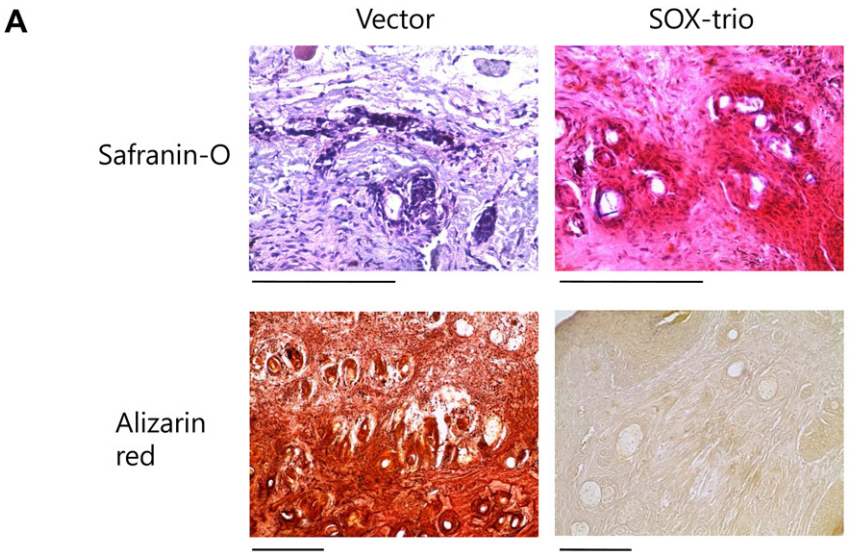
The pellets of each subset were ectopically transplanted into SCID mice to determine whether or not transfection of *SOX* genes reduces mineralization and vascular invasion of ASC pellets *in vivo*. Three-week explants of ASCs co-transfected with the *SOX trio* demonstrated still abundant proteoglycan from Safranin-O staining, and revealed a significantly reduced mineralization from Alizarin red staining compared with the control pellets. In addition, the measured GAG level was three-fold greater ($P = 0.037$) and calcium contents lower by 35% ($P = 0.037$) in ASCs co-transfected with the *SOX trio* compared with the control (Fig. 5). These results suggested that *SOX trio* transfection enhanced the *in vivo* chondrogenic potential of ASCs.

Discussion

The overall results showed that non-viral transfection of *SOX trio* greatly enhanced chondrogenesis from ASCs, while decreasing hypertrophy. These findings raise the possibility of using the method in cartilage tissue engineering. The necessity of using all three genes at the same time was also verified as statistically significant enhancement was achieved only when all three factors were transfected.

Previous studies have demonstrated the effectiveness of using the gene transfer of the *SOX trio* in enhancing chondrogenic differentiation from several cell sources. Over-expression of SOX-9 by gene transfer enhanced the expression of proteoglycan and type II collagen in a dose-dependent manner in normal and osteoarthritic AC[33]. Retroviral transduction of *SOX trio* enhanced re-expression of chondrocyte phenotypes in passaged osteoarthritic human articular chondrocytes[37]. The combination of the *SOX trio* (SOX-5, -6, and -9) has provided enough signal to induce permanent cartilage from embryonic stem cells. In the process, over-expression of the SOX trio suppressed chondrocyte hypertrophy and osteogenic differentiation, while promoting chondrogenic differentiation[30]. Our results demonstrated the effectiveness of the *SOX trio* gene transfer also in ASCs.

We successfully induced chondrogenesis using non-viral method, unlike from previous studies which used virus-mediated gene transfer[30,36,37]. While non-viral gene transfers, which include physical and chemical means, are a safe method for clinical application, these methods have been fraught with the problem of low transfection efficiency. The remarkable finding in our study was the very high efficiency of transfection (approximately 70%), as demonstrated by the FACS study. This high efficiency raises the potential for clinical applications. The half-life of a transgene is usually short in electroporation, as noted by gradual decrease in the expression of transfected genes in ASCs. In addition, as the transfected genes are transcription factors, translated protein is supposed to be rapidly destroyed. Nevertheless, greater amount of proteoglycan synthesis and still higher chondrogenic gene expression at day 21 demonstrate the effectiveness of *SOX trio* gene transfection. There is also a possibility that the transfected *SOX* genes have triggered expression of endogenous *SOX* genes. It is not certain at this point whether transient expression of chondrogenic factors as in non-viral or adenoviral gene transfer, or permanent gene expression as in retroviral gene transfer, would be more beneficial to cartilage tissue engineering. However, in view of the safety and applicability, non-viral gene transfer used in our study provides a clinically applicable method for gene transfer.



B

GAG Contents

N=3	Mean GAG / DNA (μg/μg) [95% CI]	P-value vs control
Vector (Control)	1.85 [1.5~2.21]	
SOX-trio	5.27 [4.26~6.28]	**0.037**

Calcium contents

N=3	Mean calcium / DNA (μg/μg) [95% CI]	P-value vs control
Vector (Control)	1.27 [0.61~1.92]	
SOX-trio	0.82 [0.6~1.03]	**0.037**

Fig. 5. Persistent proteoglycan and reduced calcification were observed in *in-vivo* transplanted ASCs co-transfected with *SOX trio*. Pellets were implanted in subcutaneous pockets of SCID mice for 3 weeks: Safranin-O staining and Alizarin red staining in ASCs transfected with *pEGFP-C1* without interposed gene (vector), all three genes (*SOX trio*). Scale bars are equal to 500 μm (A). The results of GAG assay and calcium assay are also presented (B).

Previous studies that applied gene therapy to enhance chondrogenesis of adult stem cells have primarily focused on gene transfer of growth factors, such as TGF-βs, IGF, and BMPs[38–44]. As growth factors are secreted outside, their effects can become uncontrollable, depending on the local concentrations. In contrast, transcription factors are the effector molecules of growth factors. Thus they are not secreted, and act inside of cells. Though the transfection of *SOX-9* was sufficient to enhance chondrogenesis from MSCs in a previous murine study[31], and effectively increased proteoglycan and type II collagen in human osteoarthritic cartilage[36], our results clearly demonstrated that SOX-5 and SOX-6 are required, as well as SOX-9, to induce effective chondrogenesis from human ASCs. Although it was out of the scope of the current study to provide an answer to this, it could be speculated that ASCs had intrinsically low activity of SOX-5 and SOX-6.

The current study is the first demonstration of the possibility of using gene transfer of *SOX trio* genes for cartilage tissue engineering from ASCs. Unlike in chondrogenic induction by growth factors[19,20], type I collagen did not increase and type X collagen decreased, while strong chondrogenic differentiation was induced by the gene transfer of the *SOX trio* in ASCs. A corollary study should be performed to substantiate the results of the ectopic model tested in this study by using an orthotopic model of cartilage healing, which will clarify whether the transient expression of the SOX trio would be enough to produce AC of durable property from ASCs *in vivo*. Nevertheless, this study provides a novel model of the *SOX trio* gene transfer to ASCs that markedly enhances chondrogenesis while suppressing hypertrophy. The results of this study will make a valuable contribution to cartilage tissue engineering from ASCs.

Author contributions

Dr. Im had full access to all of data in the study and takes responsibility for the integrity of the data analysis.

Study design: Im; Acquisition of data: Kim; Analysis and interpretation of data: Im, Kim; Manuscript preparation: Im; Statistical analysis: Im, Kim.

Conflict of interest

The authors have no conflict of interest to disclose with regard to the subject matter of this present manuscript.

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